SYNTHETIC ANTIGENS FOR THE DETECTION OF ANTIBODIES TO HEPATITIS C VIRUS

The implementation of systematic testing for hepatitis B virus (HBV) has been instrumental in eliminating this virus from the blood supply. Nevertheless, a significant number of post-transfusion hepatitis (PTH) cases still occur. These cases are generally attributable to non-A, non-B hepatitis (NANBH) virus(es), the diagnosis of which is usually made by exclusion of other viral markers.

The etiological agent responsible for a large proportion of these cases has recently been cloned (Choo, Q-L et al. Science (1988) 244:359-362) and a first-generation antibody test developed (Kuo, G. et al. Science (1989) 244:362-364). The agent has been identified as a positive-stranded RNA virus, and the sequence of its genome has been partially determined. Studies suggest that this virus, referred to subsequently as hepatitis C virus (HCV), may be related to flaviviruses and pestiviruses. A portion of the genome of an HCV isolated from a chimpanzee ($HCV_{CDC/CHI}$) is disclosed in EPO 88310922.5. The coding sequences disclosed in this document do not include sequences originating from the 5'-end of the viral genome which code for putative structural-proteins. Recently however, sequences derived from this region of the HCV genome have been published (Okamoto, H. et al., Japan J. Exp. Med. 60:167-177, 1990.). The amino acid sequences encoded by the Japanese clone HC-J1 were combined with the $HCV_{CDC/CHI}$ sequences in a region where the two sequences overlap to generate the composite sequence depicted in Figure 1. Specifically, the two sequences were joined at $glycine_{451}$. It should be emphasized that the numbering

system used for the HCV amino acid sequence is not intended to be absolute since the existence of variant HCV strains harboring deletions or insertions is highly probable. Sequences corresponding to the 5' end of the HCV genome have also recently been disclosed in EPO 90302866.0.

In order to detect potential carriers of HCV, it is necessary to have access to large amounts of viral proteins. In the case of HCV, there is currently no known method for culturing the virus, which precludes the use of virus-infected cultures as a source of viral antigens. The current first-generation antibody test makes use of a fusion protein containing a sequence of 363 amino acids encoded by the HCV genome. It was found that antibodies to this protein could be detected in 75 to 85% of chronic NANBH patients. In contrast, only approximately 15% of those patients who were in the acute phase of the disease, had antibodies which recognized this fusion protein (Kuo, G. et al. Science (1989) 244:362-364). The absence of suitable confirmatory tests, however, makes it difficult to verify these statistics. The seeming similarity between the HCV genome and that of flaviviruses makes it possible to predict the location of epitopes which are likely to be of diagnostic value. An analysis of the HCV genome reveals the presence of a continuous long open reading frame. Viral RNA is presumably translated into a long polyprotein which is subsequently cleaved by cellular and/or viral proteases. By analogy with, for example, Dengue virus, the viral structural proteins are presumed to be derived from the amino-terminal third of the viral polyprotein. At the present time, the precise sites at which the polyprotein is cleaved can only be surmised. Nevertheless, the structural proteins are likely to contain epitopes which would be useful for diagnostic purposes, both for the detection of antibodies as well as for raising antibodies which could subsequently be used for the detection

of viral antigens. Similarly, domains of nonstructural proteins are also expected to contain epitopes of diagnostic value, even though these proteins are not found as structural components of virus particles.

Brief Description of the Drawings

Figure 1 shows the amino acid sequence of the composite $HCV_{HC-J1/CDC/CHI}$

Figure 2 shows the antibody binding to individual peptides and various mixtures in an ELISA assay

Description of the Specific Embodiments

It is known that RNA viruses frequently exhibit a high rate of spontaneous mutation and, as such, it is to be expected that no two HCV isolates will be completely identical, even when derived from the same individual. For the purpose of this disclosure, a virus is considered to be the same or equivalent to HCV if it exhibits a global homology of 60 percent or more with the $HCV_{HC-J1/CDC/CHI}$ composite sequence at the nucleic acid level and 70 percent at the amino acid level.

Peptides are described which immunologically mimic proteins encoded by HCV. In order to accommodate strain-to-strain variations in sequence, conservative as well as non-conservative amino acid substitutions may be made. These will generally account for less than 35 percent of a specific sequence. It may be desirable in cases where a peptide corresponds to a region in the HCV polypeptide which is highly polymorphic, to vary one or more of the amino acids so as to better mimic the different epitopes of different viral strains.

The peptides of interest will include at least five, sometimes six, sometimes eight, sometimes twelve, usually fewer than about fifty, more usually fewer than about thirty-five, and preferably fewer than about twenty-five amino acids included within the sequence encoded by the HCV genome. In each instance, the peptide will preferably be as small as possible while still maintaining substantially all of the sensitivity of the larger peptide. It may also be desirable in certain instances to join two or more peptides together in one peptide structure.

It should be understood that the peptides described need not be identical to any particular HCV sequence, so long as the subject compounds are capable of providing for immunological competition with at least one strain of HCV. The peptides may therefore be subject to insertions, deletions, and conservative or non-conservative amino acid substitutions where such changes might provide for certain advantages in their use.

Substitutions which are considered conservative are those in which the chemical nature of the substitute is similar to that of the original amino acid. Combinations of amino acids which could be considered conservative are Gly, Ala; Asp, Glu; Asn, Gln; Val, Ile, Leu; Ser, Thr; Lys, Arg; and Phe, Tyr.

Furthermore, additional amino acids or chemical groups may be added to the amino- or carboxyl terminus for the purpose of creating a "linker arm" by which the peptide can conveniently be attached to a carrier. The linker arm will be at least one amino acid and may be as many as 60 amino acids but will most frequently be 1 to 10 amino acids. The nature of the attachment to a solid phase or carrier need not be covalent.

Natural amino acids such as cysteine, lysine, tyrosine, glutamic acid, or aspartic acid may be added to either the amino- or carboxyl terminus to provide functional groups for coupling to a solid phase or a carrier. However, other chemical groups such as, for example, biotin and thioglycolic acid, may be added to the termini which will endow the peptides with desired chemical or physical properties. The termini of the peptides may also be modified, for example, by N-terminal acetylation or terminal carboxy-amidation. The peptides of interest are described in relation to the composite amino acid sequence shown in Figure 1. The amino acid sequences are given in the conventional and universally accepted three-letter code. In addition to the amino acids shown, other groups are defined as follows: Y is, for example, NH_2 , one or more N-terminal amino acids, or other moieties added to facilitate coupling. Y may itself be modified by, for example, acetylation. Z is a bond, (an) amino acid(s), or (a) chemical group(s) which may be used for linking. X is intended to represent OH, NH_2 , or a linkage involving either of these two groups.

~~Peptide I corresponds to amino acids 1 to 20 and has the following amino acid sequences:~~

(I)

~~Y-Met-Ser-Thr-Ile-Pro-Lys-Pro-Gln-Arg-Lys-Thr-Lys-Arg-Asn-Thr-Asn-Arg-Arg-Pro-Gln-Z-X.~~

~~Peptide II corresponds to amino acids 7 to 26 and has the amino acid sequence:~~

(II)

Y-Pro-Gln-Arg-Lys-Thr-Lys-Arg-Asn-Thr-Asn-Arg-Arg-Pro-Gln-Asp-Val-Lys-Phe-Pro-Gly-Z-X.

~~Of particular interest is the oligopeptide IIA:~~

(IIA)

Y-Gln-Arg-Lys-Thr-Lys-Arg-Asn-Thr-Asn-Arg-Arg-Z-X.

~~Peptide III corresponds to amino acids 13 to 32 and has the sequence:~~

(III)

Y-Arg-Asn-Thr-Asn-Arg-Arg-Pro-Gln-Asp-Val-Lys-Phe-Pro-Gly-Gly-Gly-Gln-Ile-Val-Gly-Z-X.

~~Peptide IV corresponds to amino acid 37 to 56 and has the sequences:~~

(IV)

Y-Leu-Pro-Arg-Arg-Gly-Pro-Arg-Leu-Gly-Val-Arg-Ala-Thr-Arg-Lys-Thr-Ser-Glu-Arg-Ser-Z-X.

~~Peptide V corresponds to amino acids 49 to 68 and has the sequence:~~

(V)

Y-Thr-Arg-Lys-Thr-Ser-Glu-Arg-Ser-Gln-Pro-Arg-Gly-Arg-Arg-Gln-Pro-Ile-Pro-Lys-Val-Z-X.

~~Peptide VI corresponds to amino acid 61 to 80 and has the following sequence:~~

(VI)

Y-Arg-Arg-Gln-Pro-Ile-Pro-Lys-Val-Arg-Arg-Pro-Glu-Gly-Arg-Thr-Trp-Ala-Gln-Pro-Gly-Z-X.

~~Peptide VII corresponds to amino acids 73 to 92 and has the sequence:~~

(VII)

Y-Gly-Arg-Thr-Trp-Ala-Gln-Pro-Gly-Tyr-Pro-Trp-Pro-Leu-Tyr-Gly-Asn-Glu-Gly-Cys-Gly-Z-X.

dnaA10

A10 ~~Peptide VIII corresponds to amino acids 1688 to 1707 and has the sequence:~~

(VIII)

Y-Leu-Ser-Gly-Lys-Pro-Ala-Ile-Ile-Pro-Asp-Arg-Glu-Val-Leu-Tyr-Arg-Glu-Phe-Asp-Glu-Z-X.

dnaA11

A11 ~~Peptide IX corresponds to amino acids 1694 to 1713 and has the sequence:~~

(IX)

Y-Ile-Ile-Pro-Asp-Arg-Glu-Val-Leu-Tyr-Arg-Glu-Phe-Asp-Glu-Met-Glu-Glu-Cys-Ser-Gln-Z-X.

dnaA12

A12 ~~Peptide X corresponds to amino acids 1706 to 1725 and has the sequence:~~

(X)

Y-Asp-Glu-Met-Glu-Glu-Cys-Ser-Gln-His-Leu-Pro-Tyr-Ile-Glu-Gln-Gly-Met-Met-Leu-Ala-Z-X.

dnaA13

A13 ~~Peptide XI corresponds to amino acids 1712 to 1731 and has the sequence~~:

(XI)

Y-Ser-Gln-His-Leu-Pro-Tyr-Ile-Glu-Gln-Gly-Met-Met-Leu-Ala-Glu-Gln-Phe-Lys-Gln-Lys-Z-X.

dnaA14

A14 ~~Peptide XII corresponds to amino acids 1718 to 1737 and has the sequence:~~

(XII)

Y-Ile-Glu-Gln-Gly-Met-Met-Leu-Ala-Glu-Gln-Phe-Lys-Gln-Lys-Ala-Leu-Gly-Leu-Leu-Gln-Z-X.

~~Peptide XIII corresponds to amino acids 1724 to 1743 and has the sequence:~~

(XIII)

Y-Leu-Ala-Glu-Gln-Phe-Lys-Gln-Lys-Ala-Leu-Gly-Leu-Leu-Gln-Thr-Ala-Ser-Arg-Gln-Ala-Z-X.

~~Peptide XIV corresponds to amino acids 1730 to 1749 and has the sequence:~~

(XIV)

Y-Gln-Lys-Ala-Leu-Gly-Leu-Leu-Gln-Thr-Ala-Ser-Arg-Gln-Ala-Glu-Val-Ile-Ala-Pro-Ala-Z-X.

~~Peptide XV corresponds to amino acids 2263 to 2282 and has the sequence:~~

(XV)

Y-Glu-Asp-Glu-Arg-Glu-Ile-Ser-Val-Pro-Ala-Glu-Ile-Leu-Arg-Lys-Ser-Arg-Arg-Phe-Ala-Z-X.

~~Peptide XVI corresponds to amino acids 2275 to 2294 and has the sequence:~~

(XVI)

Y-Leu-Arg-Lys-Ser-Arg-Arg-Phe-Ala-Gln-Ala-Leu-Pro-Val-Trp-Ala-Arg-Pro-Asp-Tyr-Asn-Z-X.

~~Peptide XVII corresponds to amino acids 2287 to 2306 and has the sequence:~~

(XVII)

Y-Val-Trp-Ala-Arg-Pro-Asp-Tyr-Asn-Pro-Pro-Leu-Val-Glu-Thr-Trp-Lys-Lys-Pro-Asp-Tyr-Z-X.

Ins A20

A20 ~~Peptide XVIII corresponds to amino acids 2299 to 2318 and has the sequence:~~

(XVIII)

Y-Glu-Thr-Trp-Lys-Lys-Pro-Asp-Tyr-Glu-Pro-Pro-Val-Val-His-Gly-Cys-Pro-Leu-Pro-Pro-Z-X.

Ins A21

A21 ~~Peptide XIX corresponds to amino acids 2311 to 2330 and has the sequence:~~

(XIX)

Y-Val-His-Gly-Cys-Pro-Leu-Pro-Pro-Pro-Lys-Ser-Pro-Pro-Val-Pro-Pro-Pro-Arg-Lys-Lys-Z-X.

Of particular interest is the use of the mercapto-group of cysteines or thioglycolic acids used for acylating terminal amino groups for cyclizing the peptides or coupling two peptides together. The cyclization or coupling may occur via a single bond or may be accomplished using thiol-specific reagents to form a molecular bridge.

The peptides may be coupled to a soluble carrier for the purpose of either raising antibodies or facilitating the adsorption of the peptides to a solid phase. The nature of the carrier should be such that it has a molecular weight greater than 5000 and should not be recognized by antibodies in human serum. Generally, the carrier will be a protein. Proteins which are frequently used as carriers are keyhole limpet hemocyanin, bovine gamma globulin, bovine serum albumin, and poly-L-lysine.

There are many well described techniques for coupling peptides to carriers. The linkage may occur at the N-terminus, C-terminus or at an internal site in the peptide. The peptide may also be derivatized for

coupling. Detailed descriptions of a wide variety of coupling procedures are given, for example, in Van Regenmortel, M.H.V., Briand, J.P., Muller, S., and Plaué, S., Laboratory Techniques in Biochemistry and Molecular Biology, Vol. 19, Synthetic Polypeptides as Antigens, Elsevier Press, Amsterdam, New York, Oxford, 1988.

The peptides may also be synthesized directly on an oligo-lysine core in which both the alpha as well as the epsilon-amino groups of lysines are used as growth points for the peptides. The number of lysines comprising the core is preferably 3 or 7. Additionally, a cysteine may be included near or at the C-terminus of the complex to facilitate the formation of homo- or heterodimers. The use of this technique has been amply illustrated for hepatitis B antigens (Tam, J.P., and Lu, Y-A., Proc. Natl. Acad. Sci. USA (1989) 86:9084-9088) as well as for a variety of other antigens (see Tam, J.P., Multiple Antigen Peptide System: A Novel Design for Synthetic Peptide Vaccine and Immunoassay, in Synthetic Peptides, Approaches to Biological Problems, Tam, J.P., and Kaiser, E.T., ed. Alan R. Liss Inc., New York, 1989).

Depending on their intended use, the peptides may be either labeled or unlabeled. Labels which may be employed may be of any type, such as enzymatic, chemical, fluorescent, luminescent, or radioactive. In addition, the peptides may be modified for binding to surfaces or solid phases, such as, for example, microtiter plates, nylon membranes, glass or plastic beads, and chromatographic supports such as cellulose, silica, or agarose. The methods by which peptides can be attached or bound to solid support or surface are well known to those versed in the art.

Of particular interest is the use of mixtures of peptides for the detection of antibodies specific for hepatitis C virus. Mixtures of peptides which

are considered particularly advantageous are:

A. II, III, V, IX, and XVIII
B. I, II, V, IX, XI, XVI, and XVIII
C. II, III, IV, V, VIII, XI, XVI, and XVIII
D. II, IX, and XVIII
E. II, III, IV, and V
F. VIII, IX, XI, XIII, and XIV
G. XV, XVI, XVII, XVIII, and XIX

Antibodies which recognize the peptides can be detected in a variety of ways. A preferred method of detection is the enzyme-linked immunosorbant assay (ELISA) in which a peptide or mixture of peptides is bound to a solid support. In most cases, this will be a microtiter plate but may in principle be any sort of insoluble solid phase. A suitable dilution or dilutions of serum or other body fluid to be tested is brought into contact with the solid phase to which the peptide is bound. The incubation is carried out for a time necessary to allow the binding reaction to occur. Subsequently, unbound components are removed by washing the solid phase. The detection of immune complexes is achieved using antibodies which specifically bind to human immunoglobulins, and which have been labeled with an enzyme, preferably but not limited to either horseradish peroxidase, alkaline phosphatase, or beta-galactosidase, which is capable of converting a colorless or nearly colorless substrate or co-substrate into a highly colored product or a product capable of forming a colored complex with a chromogen. Alternatively, the detection system may employ an enzyme which, in the presence of the proper substrate(s), emits light. The amount of product formed is detected either visually, spectrophotometrically, electrochemically, or luminometrically, and is

compared to a similarly treated control. The detection system may also employ radioactively labeled antibodies, in which case the amount of immune complex is quantified by scintillation counting or gamma counting.

Other detection systems which may be used include those based on the use of protein A derived from Staphylococcus aureus Cowan strain I, protein G from group C Staphylococcus sp. (strain 26RP66), or systems which make use of the high affinity biotin-avidin or streptavidin binding reaction.

Antibodies raised to carrier-bound peptides can also be used in conjunction with labeled peptides for the detection of antibodies present in serum or other body fluids by competition assay. In this case, antibodies raised to carrier-bound peptides are attached to a solid support which may be, for example, a plastic bead or a plastic tube. Labeled peptide is then mixed with suitable dilutions of the fluid to be tested and this mixture is subsequently brought into contact with the antibody bound to the solid support. After a suitable incubation period, the solid support is washed and the amount of labeled peptide is quantified. A reduction in the amount of label bound to the solid support is indicative of the presence of antibodies in the original sample. By the same token, the peptide may also be bound to the solid support. Labeled antibody may then be allowed to compete with antibody present in the sample under conditions in which the amount of peptide is limiting. As in the previous example, a reduction in the measured signal is indicative of the presence of antibodies in the sample tested.

Another preferred method of antibody detection is the homogeneous immunoassay. There are many possible variations in the design of such

assays. By way of example, numerous possible configurations for homogeneous enzyme immunoassays and methods by which they may be performed are given in Tijssen, P., Practice and Theory of Enzyme Immunoassays, Elsevier Press, Amersham, Oxford, New York, 1985. Detection systems which may be employed include those based on enzyme channeling, bioluminescence, allosteric activation and allosteric inhibition. Methods employing liposome-entrapped enzymes or coenzymes may also be used (see Pinnaduwage, P. and Huang, L., Clin. Chem. (1988) 34/2: 268-272, and Ullman, E.F. et al., Clin. Chem. (1987) 33/9: 1579-1584 for examples).

The synthesis of the peptides can be achieved in solution or on a solid support. Synthesis protocols generally employ the use t-butyloxycarbonyl- or 9-fluorenylmethoxy-carbonyl-protected activated amino acids. The procedures for carrying out the syntheses, the types of side-chain protection, and the cleavage methods are amply described in, for example, Stewart and Young, Solid Phase Peptide Synthesis, 2nd Edition, Pierce Chemical Company, 1984; and Atherton and Sheppard, Solid Phase Peptide Synthesis, IRL Press, 1989.

Experimental

I. Peptide Synthesis

All of the peptides described were synthesized on Pepsyn K polyamide-Kieselguhr resin (Milligen, Novato, California) which had been functionalized with ethylenediamine and onto which the acid-labile linker 4-(alpha-Fmoc-amino-2',4'-dimethoxybenzyl) phenoxyacetic acid had been coupled (Rink, Tetrahedron Lett. (1987) 28:3787). t-Butyl-based side-chain protection and Fmoc alpha-amino-protection was used. The

guanidino-group of arginine was protected by the 2,2,5,7,8-pentamethylchroman-6-sulfonyl moiety. The imidazole group of histidine was protected by either t-Boc or trityl and the sulfhydryl group of cysteine was protected by a trityl group. Couplings were carried out using performed O-pentafluorophenyl esters except in the case of arginine where diisopropylcarbodiimide-mediated hydroxybenzotriazole ester formation was employed. Except for peptide I, all peptides were N-acetylated using acetic anhydride. All syntheses were carried out on a Milligen 9050 PepSynthesizer (Novato, California) using continuous flow procedures. Following cleavage with trifluoroacetic acid in the presence of scavengers and extraction with diethylether, all peptides were analyzed by C_{18} -reverse phase chromatography.

II. Detection of Antibodies to Hepatitis C Virus

A. Use of peptides bound to a nylon membrane.

Peptides were dissolved in a suitable buffer to make a concentrated stock solution which was then further diluted in phosphate-buffered saline (PBS) or sodium carbonate buffer, pH 9.6 to make working solutions. The peptides were applied as lines on a nylon membrane (Pall, Portsmouth, United Kingdom), after which the membrane was treated with casein to block unoccupied binding sites. The membrane was subsequently cut into strips perpendicular to the direction of the peptide lines. Each strip was then incubated with a serum sample diluted 1 to 100, obtained from an HCV-infected individual. Antibody binding was detected by incubating the strips with goat anti-human immunoglobulin antibodies conjugated to the enzyme alkaline phosphatase. After removing unbound conjugate by

washing, a substrate solution containing 5-bromo-4-chloro-3-indolylphosphate and nitro blue tetrazolium was added.

Positive reactions are visible as colored lines corresponding to the positions of the peptides which are specifically recognized. The reaction patterns of thirty-six different sera are tabulated in Table 1. The results shown in Table 1 are further summarized in Table 2.

B. Use of peptides in an enzyme-linked immunosorbent assay (ELISA).

Peptide stock solutions were diluted in sodium carbonate buffer, pH 9.6 and used to coat microtiter plates at a peptide concentration of 2 micrograms per milliliter. A mixture consisting of peptides II, III, V, IX, and XVIII was also used to coat plates. Following coating, the plates were blocked with casein. Fifteen HCV-antibody-positive sera and control sera from seven uninfected blood donors were diluted 1 to 20 and incubated in wells of the peptide-coated plates. Antibody binding was detected by incubating the plates with goat anti-human immunoglobulin antibodies conjugated to the enzyme horseradish peroxidase. Following removal of unbound conjugate by washing, a solution containing H_2O_2 and 3,3',5,5'-tetramethylbenzidine was added. Reactions were stopped after a suitable interval by addition of sulfuric acid. Positive reactions gave rise to a yellow color which was quantified using a conventional microtiter plate reader. The results of these determinations are tabulated in Table 3. To correct for any aspecific binding which could be attributable to the physical or chemical properties of the peptides themselves, a cut-off value was determined for each peptide

individually. This cut-off absorbance value was calculated as the average optical density of the negative samples plus 0.200. Samples giving absorbance values higher than the cut-off values are considered positive. The results for the fifteen positive serum samples are further summarized in Table 4.

While it is evident that some of the peptides are recognized by a large percentage of sera from HCV-infected individuals, it is also clear that no single peptide is recognized by all sera. In contrast, the peptide mixture was recognized by all fifteen sera and, for six of the fifteen sera, the optical densities obtained were equal to or higher than those obtained for any of the peptides individually. These results serve to illustrate the advantages of using mixtures of peptides for the detection of anti-HCV antibodies.

C. Binding of antibodies in sera from HCV-infected patients to various individual peptides and peptide mixtures in an ELISA.

Five peptides were used individually and in seven different combinations to coat microtiter plates. The plates were subsequently incubated with dilutions of fifteen HCV antibody-positive sera in order to evaluate the relative merits of using mixtures as compared to individual peptides for antibody detection. The mixtures used and the results obtained are shown in Figure 2.

In general, the mixtures functioned better than individual peptides. This was particularly evident for mixture 12 (peptides I, III, V, IX, and XVIII) which was recognized by all twelve of the sera tested. These results underscore the advantages of using mixtures of peptides

in diagnostic tests for the detection of antibodies to HCV.

D. Use of a mixture of peptides in an ELISA assay for the detection of anti-HCV antibodies.

A mixture of peptides II, III, V, IX, and XVIII was prepared and used to coat microtiter plates according to the same procedure used to test the individual peptides. A total of forty-nine sera were tested from patients with clinically diagnosed but undifferentiated chronic non A non B hepatitis as well as forty-nine sera from healthy blood donors. Detection of antibody binding was accomplished using goat anti-human immunoglobulin antibodies conjugated to horseradish peroxidase. The resulting optical density values are given in Table 5. These results indicate that the mixture of peptides is not recognized by antibodies in sera from healthy donors (0/49 reactives) but is recognized by a large proportion (41/49, or 84%) of the sera from patients with chronic NANBH. These results demonstrate that the peptides described can be used effectively as mixtures for the diagnosis of HCV infection.

E. Detection of anti-HCV antibodies in sera from patients with acute NANB infection using individual peptides bound to nylon membranes and a mixture of peptides in an ELISA assay, and comparison with a commercially available kit.

Peptides were applied to nylon membranes or mixed and used to coat microtiter plates as previously described. The peptide mixture consisted of peptides II, III, V, IX, and XVIII. Sera obtained from twenty-nine patients with acute non-A, non-B hepatitis were then

tested for the presence of antibodies to hepatitis C virus. These same sera were also evaluated using a commercially available kit (Ortho, Emeryville, CA, USA).

The results of this comparative study are given in Table 6. In order to be able to compare the peptide-based ELISA with the commercially available kit, the results for both tests are also expressed as signal to noise ratios (S/N) which were calculated by dividing the measured optical density obtained for each sample by the cut-off value. A signal-to-noise ratio greater or equal to 1.0 is taken to represent a positive reaction. For the commercially available kit, the cut-off value was calculated according to the manufacturer's instructions. The cut-off value for the peptide-based ELISA was calculated as the average optical density of five negative samples plus 0.200.

The scale used to evaluate antibody recognition of nylon-bound peptides was the same as that given in Table 1. Of the twenty-nine samples tested, twenty-five (86%) were positive in the peptide-based ELISA and recognized one or more nylon-bound peptides. In contrast, only fourteen of the twenty-nine sera scored positive in the commercially available ELISA. These results serve to illustrate the advantages of using peptide mixtures for the detection of anti-HIV antibodies as well as the need to include in the mixtures peptides which contain amino acid sequences derived from different regions of the HCV polyprotein.

Table 1. Recognition of peptides bound to nylon membranes by sera from persons infected by HCV.

PEPTIDE

Serum nr.	I	II	III	IV	V	VI	VII	VIII	IX
1			3	1				1	
2									
3	1	0.5	2	1		0.5		2	0.5
4									
6			2	1	0.5				
7	0.5	1	2	1	0.5			3	2
8	0.5	1	3	1	1		1	1	
10		1	0.5					3	1
13	0.5	0.5	2		0.5				
15				0.5				2	1
16	2	1	0.5	0.5	1	0.5		2	0.5
18	1	1	3	0.5		2	0.5		
23	0.5		1	1			0.5		
24	1	0.5	2	1	0.5	0.5	0.5	2	
25			1	0.5				2	0.5
26								1	
27	0.5	0.5	1					3	2
29		0.5	3	2	1	1	0.5		
30		0.5	0.5	1	1	0.5			
31		1	0.5						
32		1	2						
33								0.5	
34		1	1	1				3	1
35	1	1	2	1	1	1	0.5		
36	1		2	1		1			
37	1	1							
44		1	2	1	0.5				
46		0.5	2	0.5	0.5		0.5	2	
47		0.5		0.5		0.5		1	
48	1	2		2			0.5	2	
49		1	1	0.5	0.5	0.5			
50		1	2	1				2	0.5
51			2	0.5	0.5		0.5		
52			2	0.5			0.5		
54			2		0.5	0.5	1	0.5	
56	ND	ND	ND	ND	ND	ND	ND	2	

Blank: no reaction; 0.5: weakly positive; 1: clearly positive; 2: strong reaction; 3: intense reaction; ND: not determined

TABLE 1 continued

X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII	XIX
	0.5					2	2	1	1
	0.5		1	2		2	1		
1	0.5								
			1						
						2			
2	1		1		2		0.5	1	1
2	1		1			1	1	0.5	
1				0.5	2	2		2	2
1			1	0.5		0.5			
0.5				1		0.5			
1					2	2	1	2	
						1	0.5		
1	0.5		0.5		0.5				
						1			
0.5	2		1	1		2			
						0.5			
						1	2	1	0.5
2	1		1	1	2	2	1	1	1
0.5	0.5				0.5				
1	0.5				1	1			
1	0.5								
								0.5	
						1	1	0.5	
					2		0.5		
								1	1
			0.5		1	1		1	0.5
0.5	0.5		0.5				1		
1	1		1			1	1	0.5	0.5
						1	1	0.5	
0.5									
1	1		1			1	1	1	
	0.5	1	2	1					

Table 2. Summary of antibody binding to nylon-bound HCV peptides by sera from infected patients.

Peptide	No. reactive sera	% reactive sera
I	13/35	37
II	22/35	63
III	27/35	77
IV	24/35	69
V	14/35	40
VI	11/35	31
VII	11/35	31
VIII	19/36	53
IX	9/36	25
X	17/36	47
XI	15/36	42
XII	1/36	3
XIII	13/36	36
XIV	7/36	19
XV	9/36	25
XVI	20/36	56
XVII	14/36	39
XVIII	14/36	39
XIX	8/36	22

Table 3. Comparison of Individual Peptides in an ELISA Assay for the Detection of Antibodies to HCV.

sample ident	peptide								
	I	II	III	IV	V	VI	VII	VIII	IX
1	0.786	1.119	1.284	0.265	0.042	0.04	0.05	0.571	0.659
2	0.044	0.039	0.11	0.041	0.037	0.038	0.039	0.479	0.78
3	0.815	0.944	0.825	0.399	0.654	0.487	0.32	0.705	0.965
7	1.122	1.23	0.588	0.682	0.659	0.182	0.107	0.907	1.42
8	1.155	1.159	1.2	0.508	1.272	0.433	0.623	0.61	0.863
10	1.089	1.236	1.083	0.044	0.508	0.042	0.073	1.49	1.529
11	0.048	0.051	0.476	0.052	0.119	0.039	0.1	0.634	0.711
15	0.224	0.602	0.813	0.093	0.068	0.077	0.147	0.807	1.225
23	0.62	0.8	0.924	0.568	0.759	0.442	0.683	0.089	0.121
24	1.042	1.132	1.026	0.518	0.916	0.302	0.253	1.013	1.364
49	0.624	0.73	0.884	0.171	0.372	0.055	0.04	0.084	0.064
13	0.76	0.857	0.815	0.087	0.422	0.098	0.045	0.473	0.489
31	0.84	1.114	0.445	0.672	0.046	0.041	0.042	0.184	0.15
47	1.303	1.53	1.236	0.751	0.83	0.629	0.073	0.545	0.739
56	1.169	1.301	1.364	1.269	1.374	0.85	1.066	1.45	1.523
bd A28	0.054	0.043	0.139	0.045	0.135	0.042	0.041	0.086	0.115
bd A169	0.041	0.042	0.134	0.044	0.038	0.04	0.041	0.061	0.07
bd A170	0.04	0.044	0.117	0.04	0.036	0.04	0.04	0.081	0.05
bd A171	0.041	0.046	0.148	0.043	0.037	0.045	0.045	0.077	0.065
bd A166	0.047	0.046	0.124	0.044	0.038	0.042	0.041	0.056	0.066
bd A165	0.041	0.046	0.123	0.043	0.035	0.051	0.042	0.051	0.091
AVG	0.044	0.045	0.131	0.043	0.053	0.043	0.042	0.069	0.076
STD	0.005	0.002	0.011	0.002	0.037	0.004	0.002	0.013	0.021
cut off	0.109	0.101	0.214	0.099	0.214	0.105	0.098	0.158	0.189

TABLE 3 continued

X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII	XIX
0.048	0.04	0.043	0.068	0.044	0.041	1.063	0.956	1.383	1.346
0.169	0.563	0.039	0.042	0.515	0.039	0.64	0.319	0.154	0.49
0.468	0.668	0.041	0.093	0.341	0.043	0.292	0.038	0.046	0.038
0.663	0.646	0.041	0.235	0.068	0.575	0.042	0.041	0.872	1.271
0.752	1.175	0.046	0.42	0.102	0.068	0.552	0.671	0.417	0.058
0.689	0.834	0.041	0.044	0.314	0.793	0.886	0.037	1.335	1.356
0.199	0.967	0.125	0.454	0.088	0.111	0.274	0.093	0.838	0.065
0.315	0.688	0.046	0.154	0.202	0.065	0.372	0.097	0.155	0.077
0.422	0.896	0.041	0.049	0.101	0.068	0.311	0.038	0.052	0.05
0.236	0.397	0.054	0.123	0.076	0.051	0.418	0.053	0.1	0.085
0.209	0.731	0.044	0.113	0.039	0.044	0.299	0.038	0.192	0.041
0.529	0.735	0.043	0.044	0.186	0.043	0.086	0.037	0.066	0.04
0.255	0.69	0.041	0.04	0.061	0.136	0.292	0.038	0.224	0.501
0.044	0.041	0.041	0.041	0.498	0.04	0.268	0.042	1.288	1.206
0.079	1.069	0.058	0.568	0.038	0.039	0.218	0.036	0.087	0.039
0.044	0.042	0.044	0.052	0.043	0.043	0.307	0.042	0.045	0.061
0.043	0.042	0.041	0.04	0.041	0.041	0.255	0.038	0.056	0.042
0.04	0.039	0.04	0.038	0.038	0.144	0.292	0.036	0.058	0.039
0.043	0.041	0.043	0.039	0.04	0.045	0.286	0.037	0.05	0.04
0.041	0.041	0.042	0.04	0.041	0.041	0.207	0.039	0.046	0.041
0.041	0.04	0.042	0.039	0.043	0.039	0.253	0.034	0.06	0.098
0.042	0.041	0.042	0.041	0.041	0.059	0.267	0.038	0.053	0.054
0.001	0.001	0.001	0.005	0.002	0.038	0.033	0.002	0.006	0.021
0.095	0.094	0.095	0.106	0.097	0.223	0.416	0.084	0.121	0.167

Table 4 Summary of antibody-binding to individual peptides in an ELISA assay.

Peptide	No. reactive sera	% reactive sera
I	13	87
II	13	87
III	14	93
IV	10	67
V	10	67
VI	7	47
VII	8	53
VIII	13	87
IX	12	80
X	13	87
XI	13	87
XII	1	7
XIII	7	47
XIV	8	53
XV	2	13
XVI	5	33
XVII	4	27
XVIII	10	67
XIX	6	40

Table 5 Use of a peptide mixture for the detection of antibodies to HCV in sera from chronic NANBH patients and comparison to sera from healthy blood donors.

Chronic NANB Sera		Control Sera	
Serum nr.	Optical Density	Serum nr.	Optical Density
101	0.041	1	0.049
102	1.387	2	0.047
103	1.578	3	0.049
104	1.804	4	0.046
105	1.393	5	0.049
107	1.604	6	0.045
108	1.148	7	0.043
109	1.714	8	0.053
110	1.692	9	0.049
112	0.919	10	0.047
113	1.454	11	0.060
114	0.936	12	0.044
115	0.041	13	0.049
116	1.636	14	0.051
118	1.242	15	0.056
119	1.568	16	0.050
120	1.290	17	0.049
121	1.541	18	0.055
122	1.422	19	0.054
123	1.493	20	0.058

124	1.666	21	0.050
125	1.644	22	0.044
126	1.409	23	0.043
127	1.625	24	0.045
128	1.061	25	0.046
129	1.553	26	0.049
130	1.709	27	0.050
131	0.041	28	0.047
132	0.044	29	0.050
133	1.648	30	0.053
134	-0.043	31	0.051
135	1.268	32	0.053
136	1.480	33	0.055
138	0.628	34	0.064
139	0.042	35	0.063
140	0.040	36	0.057
141	0.039	38	0.048
142	1.659	39	0.045
143	1.457	40	0.046
144	0.722	41	0.046
145	1.256	42	0.051
146	0.373	43	0.057
147	1.732	44	0.050
148	1.089	45	0.050
149	1.606	46	0.045
150	1.725	47	0.041
151	1.449	48	0.064
154	1.639	49	0.040
155	1.775	50	0.036

Table 6. Comparison of anti-HCV antibody detection by nylon-bound peptides, a peptide-based ELISA, and a commercially available kit.

Serum nr.	Nylon-bound peptides I	III	IV	V	VI	VIII	XI	XIV	XV	XVI	XVIII
191	0	0	0	0	0	0	0	0	0	0	0
192	0	0	0	0	0	0	0	0	0	0	0
193	0	0	0	0	0	0	0	0	0	0	0
194	0	0	0	0	0	0	0	0	0	0	0
195	1	2	2	3	0	0	0.5	0.5	1	3	1
196	1	2	1	2	0.5	0.5	0.5	0.5	0.5	2	0
197	1	2	1	2	0	0.5	0.5	0.5	1	2	0
198	1	2	2	2	0	0	0	0	1	2	0
211	0.5	1	0.5	0.5	0	2	2	0	2	0	1
213	0	0	0	1	0	0	0	0	0	0	0
214	0	0	0	1	0	0	0	0	0	0	0
215	0	0	0	1	0	0	0	0	0	0	0
216	0	0	0	0.5	0	0	0	0	0	0	0
217	0	0	0	1	0	0	0	0	0	0	0
219	0.5	1	1	2	1	0.5	1	0	0.5	0.5	1
220	0.5	1	1	2	1	0.5	1	0	0.5	0.5	1
221	0	0	0	0.5	0	0	0	0	0	0	0
222	1	1	1	1	0	0	2	0.5	0.5	0	0
223	1	1	1	1	0	0	3	0.5	0.5	0	0
224	1	1	2	1	0	0.5	3	0.5	0.5	0	0
225	0	0	0	0	0	0.5	0.5	0.5	0	0	2
226	0.5	0	0	0	0	2	3	2	0.5	0.5	3
227	0	0	0	0	0	2	2	0.5	0.5	0.5	2
228	0.5	0	0.5	0.5	0	2	2	2	0	0	2
234	0.5	0.5	0	0.5	0	0	3	1	3	1	3
235	0	0	0	0.5	0	0	0	0	0	0	0
236	0	0	0	0.5	0	0	0	0	0	0	0
237	0	0	0	1	0	0	0	0	0	0	0
238	0	0	0	1	0	1	1	0	0	0	0

0: no reaction; 0.5: weakly positive; 1: clearly positive;
2: strong reaction; 3: intense reaction;

Table 6 continued

Serum nr.	Optical density Peptide ELISA	S/N	Optical density Commercial ELISA	S/N
191	0.045	0.18	0.295	0.47
192	0.042	0.17	0.289	0.46
193	0.039	0.16	0.197	0.32
194	0.044	0.18	0.183	0.29
195	1.692	6.77	3.000*	4.82*
196	1.569	6.28	0.386	0.62
197	1.523	6.09	0.447	0.72
198	1.578	6.31	0.354	0.57
211	1.606	6.42	3.000*	4.82*
213	0.369	1.48	0.127	0.20
214	0.444	1.78	0.101	0.16
215	0.637	2.55	0.101	0.16
216	0.812	3.25	0.092	0.15
217	1.320	5.28	0.875	1.40
219	1.547	6.19	3.000*	4.82*
220	1.536	6.14	3.000*	4.82*
221	1.428	5.71	0.327	0.52
222	1.362	5.45	3.000*	4.82*
223	1.316	5.26	3.000*	4.82*
224	1.304	5.22	3.000*	4.82*
225	1.178	4.71	2.398	3.85
226	1.286	5.14	3.000*	4.82*
227	1.335	5.34	3.000*	4.82*
228	1.400	5.60	3.000*	4.82*
234	1.481	5.92	3.000*	4.82*
235	0.351	1.40	0.257	0.41
236	0.475	1.90	0.245	0.39
237	1.134	4.54	0.351	0.56
238	1.096	4.38	1.074	1.72
	Cut-off: 0.250		Cut-off: 0.623	

* O.D. exceeded 3.000 and was out of range. The values given are therefore minimum values.